EVIDENCE FOR A SECOND MSH-CONTROLLED POINT OF REGULATION IN THE MELANIN BIOSYNTHETIC PATHWAY: CYTOTOXICITY STUDIES

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Evidence for a Second MHC-Controlled Level of Recognition in the Murine Allogeneic Defense Cytotoxicity System

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A Thesis Submitted in Partial Fulfillment
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Evidence for a Second MSH-Controlled Point of Regulation in the Melanin Biosynthetic Pathway: Cytotoxicity Studies

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to my parents

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INTRODUCTION

A. Background

The biochemical effects of MSH

Approximately forty years ago, the Cloudman S-91 melanoma was established as a transplantable tumor in DBA/2J mice (1). Yasmura *et*. *al*. in 1966 were the first to adapt the tumor cells to culture (2). The biochemical and morphological response of cultured Cloudman melanoma cells to melanotropin (MSH) was described by Wong and Pawelek (3,4). They observed dramatic increases in tyrosinase activity and melanin content as well as increased generation time when cells were exposed to the hormone. Previous studies *in vivo* had produced similar results. Geschwind and Huseby saw darkening of coat color and marked increases in tyrosinase activity following MSH injections into mice (5). When mice with B-16 melanomas were injected with MSH, Lee *et*. *al*. reported an increase in both tyrosinase activity and melanogenesis of the tumor (6).

Bitensky and Demopoulos found that broken-cell preparations of Cloudman mouse melanoma had adenylyl cyclase activity which responded to both α and β MSH (7,8), indicating that MSH might act through adenosine 3',5'-monophosphate

(cyclic AMP) as a second messenger. Support for this proposed mechanism of action came from further studies in which cyclic AMP or its analogue, $N^6,O^{2'}$-dibutyryl adenosine 3', 5'-monophosphate (dibutyryl cyclic AMP), was shown to mimic the effects of MSH on S-91 cells in culture (4,9). In addition, Pawelek <u>et</u>. <u>al</u>. reported a 5 to 50 fold rise in intracellular cyclic AMP within 10 minutes of addition of MSH (10).

Later studies have reinforced the role of cyclic AMP in cellular regulation of both pigmentation and proliferation. Firstly, a link between cyclic AMP levels and tyrosinase activity in Cloudman S-91 melanoma cells has been postulated based upon the following observations: (1) In response to MSH, maximum levels of both occur during the G_2 phase of the cell cycle, with the tyrosinase peak following the cyclic AMP peak by 8 hr (11); (2) The addition of a partially purified cyclic AMP-dependent protein kinase to a crude cell extract from non-MSH treated cells resulted in a several fold increase in tyrosinase activity (12). Secondly, a link between growth characteristics and cyclic AMP has been made based upon studies showing: (1) growth inhibition from high levels of dibutyryl cyclic AMP (8×10^{-4}M) (13) or cyclic AMP (1-10 $\times 10^{-3}$M) (14); (2) stimulation of proliferation with low levels of dibutyryl cyclic AMP (10^{-5}M) (13); and (3) a major cyclic AMP-dependent protein kinase isolated from cells of a

mutant S-91 line (characterized by dependence upon MSH for growth) had a somewhat higher activation constant for cyclic AMP as compared to the wild type (15). This could explain the mutant cells' requirement of elevated levels of cyclic AMP for growth.

Regulation of melanin synthesis

While advances have been made in the understanding of the regulation of pigmentation, the accepted pathway of melanin synthesis has remained unchallenged for several decades (16,17) (Fig. 1). It has been commonly thought that both normal and malignant mammalian melanocytes synthesize melanin in a pathway controlled exclusively by the enzyme tyrosinase (monophenol monoxygenase; monophenol, dihydroxyphenylalanine: oxygen oxidoreductase, EC 1.1418.8). There remains some controversy as to whether in murine melanoma the two initial reactions of (1) the oxidation of tyrosine to L-3,4-dihydroxyphenylalanine (dopa) and (2) the oxidation of dopa to dopa quinone are catalyzed by a single enzyme tyrosinase (18,19,20,21), or whether there are two separate enzymes, i.e. a peroxidase for the first conversion and a dopa oxidase for the second conversion (22,23,24). Despite these differences, it has been generally agreed that once tyrosine was converted to dopa quinone, the subsequent oxidation-reduction

reactions proceeded spontaneously with no further regulatory control (25).

In the past year, however, Logan and Weatherhead have observed a post-tyrosinase inhibition of melanin synthesis by melatonin in hamster hair follicles (26). Recently, Korner and Pawelek reported the isolation of a factor from murine melanomas which catalyzes the conversion of dopachrome to 5,6-dihydroxyindole-2-carboxylic acid (27) (Fig. 1). In addition, another regulatory factor has been isolated that inhibits the spontaneous conversion of dopachrome to melanin (28). Treatment of cells with MSH results in removal of this inhibitory factor. Therefore, recent evidence suggests that, contrary to previous thought, there are multiple points of regulation in the pathway of melanin biosynthesis.

Cytotoxicity of melanin precursors

In 1963, Hochstein and Cohen were the first to comment upon the potential cytotoxicity of the intermediates of the melanin biosynthetic pathway (29). These precursors are polyphenolic and quinone compounds and numerous studies have shown that related compounds, such as hydroquinone (30,31,32, 33) and hydroxyanisole (34,35), produce selective destruction of pigmented melanocytes. Two possible mechanisms of toxicity were proposed (29): (1) the formation of hydrogen

peroxide as a result of the autoxidation of phenolic intermediates, and a subsequent damage to cellular integrity (36) or (2) quinone intermediates or products causing oxidative inactivation of sulfhydryl-dependent enzymes, essential in both energy metabolism (37) and DNA synthesis (38).

Graham et. al. postulated that phenolic compounds whose cytotoxicity correlated with their rate of oxidation and oxidative potentials formed hydrogen peroxide and free radicals from autoxidation (mechanism one) (39). These investigators also examined the quinone intermediates which result from the oxidation of tyrosine for their ability to inhibit DNA polymerase α, a sensitive test for sulfhydryl reagents (mechanism two) (40). The greatest affinity for the enzyme was shown by a dopa quinone analogue, leading to the conclusion that dopa quinone was the significant toxic metabolite.

It has been proposed by Lerner that normal and malignant melanocytes possess a mechanism which protects against the toxic metabolites being produced continuously as a result of melanogenesis (41). However, this protection was viewed as somewhat labile. The chance that these melanin precursors could cause cellular self-destruction would be increased if melanin synthesis were stimulated. This could aid in explaining the following observations in humans: the appearance of vitiligo in areas of the body which are normally hyperpigmented, the occurence of halo nevi and halo metastases

from melanoma, and the association of vitiligo with Addison's disease and melanoma (41).

Pawelek _et_. _al_. were interested in testing the hypothesis that the cytotoxicity of melanin precursors would be increased if melanin synthesis were increased. Tyrosine (5 mM), the initial precursor in the melanin pathway, and its analogue N-acetyl tyrosine (50 mM) were found to be selectively toxic toward melanotic melanoma cells (10). While the melanotic cells died after four days of exposure, amelanotic variants, characterized by low basal tyrosinase activity (42), displayed a decline in growth rate, but no mortality (10). If DBA/2J mice with Cloudman melanoma tumors were fed a diet high in tyrosine (33% wt/wt) for four weeks, a 2-5 fold reduction in tumor size was recorded in comparison to the control. When these animals were given daily injections of MSH, the animals on the control diet had increased tumor size, while the animals on the tyrosine diet had even smaller tumors than those not injected with the hormone (10). These results could be explained by the stimulation of melanin synthesis by MSH, thereby increasing the sensitivity of the tumor cells to the cytotoxic effects of tyrosine.

Subsequently, both melanotic Cloudman melanoma cells and amelanotic mouse L-cell fibroblasts were exposed to 21 of the naturally occurring amino acids for a period of six days. Only tyrosine (5 mM), dopa (0.1 mM), and tryptophan

(5 mM) resulted in selective cytotoxicity in the pigmented cell line (25). Since tyrosine and dopa are the principal amino acids that serve as precursors in the melanin biosynthetic pathway, their selective toxicity was expected. However, Costa <u>et</u>. <u>al</u>. have observed the incorporation of tryptophan into melanin in Harding-Passey mouse melanoma (43), which would explain the toxicity of this amino acid. In addition, pretreatment of cells with MSH increased melanin synthesis and as predicted, rendered the melanoma cells more susceptible to the toxicity of tyrosine (25) and dopa (44). Treatment of cells with phenylthiourea, a potent inhibitor of tyrosinase, provided protection from the toxicity of tyrosine (25).

Studies by Wick <u>et</u>. <u>al</u>. have shown that both dopa (45) and its analogue, 6-hydroxydopa (46), exert selective inhibition of growth in pigmented cell lines. Melanotic S-91 melanoma cells, exposed to dopa (6 mM) for one hour, showed a 60% decrease in cell number when counted 48 hr later. In comparison, human melanoma cells showed a decrease of 35% while dopa had no effect on the amelanotic S-91 cells and the mouse fibroblasts (45). These findings are similar to those made by Pawelek (25) and substantiate the selective cytotoxicity of dopa, the first intermediate in the melanin pathway.

Wick has also observed that the toxicity of dopa and

6-hydroxydopa paralled the ability of various cell lines to incorporate dopa (47). For example, the melanotic melanoma cells incorporated dopa up to 60 times that of amelanotic cell lines. On the other hand, tyrosine showed no selective uptake. The inability to detect a selective accumulation of tyrosine in pigmented cells was explained by the finding that an estimated 95% of the tyrosine incorporated into cells was used for protein synthesis. The explanation for dopa's selective incorporation was its lack of alternate routes of metabolism other than melanin synthesis.

Cytotoxicity studies reported in the past year by Pawelek and Lerner have demonstrated the potent toxicity of 5,6-dihydroxyindole, a melanin precursor two steps removed from the final product, melanin (44). When a comparison of dose-responses was made, 5,6-dihydroxyindole was more toxic, as reflected by inhibition of thymidine incorporation, than dopa. However, 5,6-dihydroxyindole exhibited a wider range of cytotoxicity than dopa, killing both pigmented cells and mouse fibroblasts (44). To date, three of the melanin pathway intermediates, tyrosine, dopa, and 5,6-dihydroxyindole, have been shown to inhibit cellular growth of melanomas.

B. Hypothesis

The work in this thesis was based upon three observa-

tions: (1) When S-91 melanoma cells are pretreated with MSH, they display increased sensitivity to the cytotoxic effects of the melanin precursors tyrosine and dopa (25,44). The explanation for this effect was an increase in tyrosinase activity, leading to an increase in the rate of production of melanin precursors. (2) Extracts from cells not exposed to MSH contain a block in the melanin pathway and are unable to convert dopachrome to melanin even though conversion of dopachrome to melanin can occur spontaneously within an hour (28). (3) Pretreatment of the cells with MSH removed this block (28).

The studies described here provide evidence for a more distal site of regulation in the melanin pathway than tyrosinase. The hypothesis was that the above mentioned blocking factor could influence the cytotoxicity of dopachrome. Dopachrome is a melanin precursor whose conversion to melanin is independent of tyrosinase, but dependent upon the absence of this blocking factor. Theoretically, treatment of the cells with MSH would remove the inhibitory factor and allow the intracellular concentration of toxic dopachrome derivatives to increase. The major finding of this study is that the cytotoxicity of dopachrome is increased when the melanoma cells are pretreated with MSH. In conclusion, this inhibitory factor appears to protect cells from the cytotoxic effects of melanin precursors, and it is removed when the cells are exposed to MSH.

MATERIALS AND METHODS

Cell lines

Cloudman S-91 murine melanoma cells, clone M-3, CCL-53.1, were obtained from the American Type Cell Culture Repository (48). Isogenous pigmented cell lines were established by sub-cloning in soft agar (42,49), including the PS1-HGPRT-1 (hypoxanthine-guanine phosphoribosyl transferase deficient) cell line here referred to as wild type. This line was originally isolated because of its resistance to 6-thioguanine and 8-azaguanine following ethylmethane sulfonate treatment of the parental cell line (13). The phenotype of the PS1-HGPRT-1 cell line was one of low basal tyrosinase activity which increased 5-50 fold after 15 hr of exposure to MSH (12).

In addition to the wild type, two clones of melanoma cells resistant to the inhibitory effects of MSH on growth were used in these experiments (50). One clone was melanotic in the presence of MSH and was designated <u>mel-1</u>. A second clone was amelanotic in the presence of MSH and was called <u>amel-1</u>. In the absence of MSH, both lines were amelanotic and had tyrosinase activity $\leqslant 5\%$ of that found in the wild type. In the presence of MSH, only the <u>mel-1</u> line showed a significant rise in tyrosinase activity (50).

Cell Culture

Cells were grown as monolayers in Corning tissue culture flasks in Ham's F10 nutrient medium (51) supplemented with 2% fetal calf serum (Flow Labs), 10% horse serum (Flow Labs), 1.2 mg/ml of sodium bicarbonate, 100 U/ml of penicillin, and 100 µg/ml of streptomycin. (Neither antibiotic affected pigment formation (52).) Cells were incubated at 37°C in a humidified incubator with 5% CO_2-95% air. Fresh culture medium was added three times per week. Subculturing involved removal of culture medium and addition of Joklik's medium (Difco) containing ethylenediaminetetracetic acid (EDTA) (5 mM) for 10 min at 25°C. This was followed by centrifugation of detached cells, resuspension in fresh medium, and inoculation into flasks.

Preparation of dopachrome

Dopachrome was synthesized by the addition of ice cold dopa (0.5 or 1.0 mg/ml 0.1 M potassium phosphate buffer pH 6.8) to solid Ag_2O [30 mg Ag_2O : 1 mg dopa] (17) for approximately one min or until the desired red-orange color was obtained. The mixture was shaken during this period, then filtered through a Gelman Acrodisc Disposable Filter 4192 with 0.2 µm diameter pores. After 15-20 min on ice, the optical density at 475 mµ was measured on a spectrophotometer.

(Maximum absorption for dopachrome = 305 mμ, 475 mμ) Using the formula optical density (OD) = molecular extinction coefficient (ϵ) x concentration in moles per liter (C), the concentration of dopachrome was calculated. ($\epsilon_{\text{dopachrome}}$ = 3520, pH 6.7 (39)) L-dopa was obtained from Hoffman-LaRoche.

^{3}H-Thymidine incorporation

Effect of dopa and dopachrome - 1 x 10^5 wild type melanoma cells in 5 ml media were seeded into 25 cm^2 Corning tissue culture flasks. 24 hr later, the medium was removed and replaced with fresh medium containing either 0.1 M potassium phosphate buffer pH 6.8, dopa (2.5 x 10^{-4}M), or dopachrome (7 x 10^{-5}M), as well as [^{3}H] thymidine [methyl-^{3}H] (New England Nuclear), specific activity of 30 Ci/mmol in a concentration of 1 μCi/ml. The flasks were incubated at 37°C. At times 30 min, 1, 2, and 4 hr, the amount of ^{3}H-thymidine incorporated into acid-precipitable material was determined by the following method.

The medium containing the ^{3}H-thymidine was removed. The cells were then harvested in 10 ml of EDTA (5 mM) in Ca^{++}-Mg^{++} free Tyrode salt solution with 0.5 mg/ml non-radioactive thymidine. A 1 ml sample of the suspended cells was taken and counted in a Coulter counter. The remaining 9 ml were centrifuged (1500g, 10 min at 4°C), resuspended in 4 ml ice-

cold 0.9% NaCl with 0.5 mg/ml thymidine, and precipitated with 4 ml ice-cold 10% trichloroacetic acid containing 0.5 mg/ml thymidine. After 15 min at 4°C, the acid precipitates were filtered onto 0.45 µm Millipore filters and were washed 5 times with 5 ml ice-cold 5% trichloroacetic acid containing 0.5 mg/ml thymidine. Two ml of scintillation fluid were added to the filters and the samples were counted in a Beckman scintillation counter 16 hr later, at which time the filters had dissolved. ^{3}H-thymidine incorporation into acid-precipitable material was expressed as c.p.m./10^5 cells.

In order to construct a dose-response curve, wild type cells were seeded as above. 24 hr later, fresh medium was added containing either 0.1 M potassium phosphate buffer pH 6.8, dopa (1×10^{-3}M to 1×10^{-5}M), or dopachrome (4×10^{-4}M to 3×10^{-6}M), in addition to ^{3}H-thymidine (1 µCi/ml). The cells were harvested at 90 min and the acid-precipitable material was assayed.

Effect of pretreatment with MSH - Wild type and mel-1 melanoma cells at 1×10^5 cells per flask were grown in 5 ml medium. The medium contained either no additions or MSH (2×10^{-7}M) and 3-isobutyl-1-methylxanthine (MIX) (1×10^{-4}M). MIX is a potent inhibitor of cyclic nucleotide phosphodiesterase (53) and has been shown to increase the intracellular content of both melanin and cyclic AMP in B-16 melanoma cells

(54). Phosphodiesterase inhibitors, including theophylline and MIX, have been used to potentiate the effects of MSH (44, 50,55). Cells were incubated for 24 or 48 hr. The medium was then replaced with fresh medium containing either phosphate buffer or dopachrome (4-8 x 10^{-5}M), as well as ^{3}H-thymidine (1 μCi/ml). Cells from triplicate flasks were harvested at 90 min and acid-precipitable material assayed for ^{3}H-thymidine incorporation as above. MSH was prepared by Drs. A. Lerner and S. Lande. MIX was obtained from Aldrich Chem Co.

Growth inhibition

Effect of melanin precursors - Wild type, *mel-1*, or *amel-1* melanoma cells were seeded at 1 x 10^5 cells in 2.5 ml medium. After approximately 3 hr, when the cells had attached, 2.5 ml of medium was added, containing either no additions or MSH and MIX. The final concentrations of MSH and MIX were 2 x 10^{-7}M and 1 x 10^{-4}M, respectively. The cells were incubated for 48 hr at 37°C. The medium was then removed and replaced with fresh medium containing either 0.1 M potassium phosphate buffer, phosphate buffer previously mixed with solid Ag_2O and filtered, dopa (1 x 10^{-3}M to 1 x 10^{-5}M), or dopachrome (5 x 10^{-4}M to 5 x 10^{-6}M). Following the mixture of phosphate buffer and solid Ag_2O, no change in color

was noted. After 2 hr of incubation at 37°C, the medium was removed and the cells were washed with 5 ml of fresh medium and then reincubated with 5 ml fresh medium for 0, 24, or 48 hr. Media and detached cells were removed and 10 ml of EDTA (5 mM) in Ca^{++}-Mg^{++} free Tyrodes solution was added for 10 min at 37°C. Cells were harvested and counted in a Coulter counter. After 24 hr, a 10% reduction in cell number was observed in the cultures treated with the phosphate buffer that had been previously mixed with Ag_2O in comparison to the control. This effect was non-specific, i.e. it was seen in both MSH- and non-MSH-treated amel-1 and mel-1 cells.

The detached cells were also counted, inoculated with fresh medium into new flasks, and studied with 0.4% trypan blue stain.

Effect of MSH versus MIX - 1×10^5 amel-1 or mel-1 cells were seeded in 2.5 ml medium as above. Three hr later, 2.5 ml medium containing either no additions, MIX, MSH, or MSH and MIX were added. The final concentrations of MSH and MIX were 1×10^{-7}M and 5×10^{-5}M, respectively. The cells were incubated for 48 hr. Medium was then removed and replaced with fresh medium containing either dopachrome (4-8×10^{-5}M) or phosphate buffer previously mixed with Ag_2O. After 2 hr of incubation, the medium was removed and the cells were washed with fresh medium and reincubated for 24

hr. Cells were harvested as above and counted.

<u>Effect of dibutyryl cyclic AMP</u> - 1 x 10^5 <u>mel-1</u> cells were inoculated in 2.5 ml medium as described previously. Three hr later, 2.5 ml medium containing either no additions, MSH and MIX, or dibutyryl cyclic AMP was added. Final concentrations of MSH, MIX, and dibutyryl cyclic AMP were 2 x 10^{-7}M, 5 x 10^{-5}M, and 1 x 10^{-3}M, respectively. After 48 hr, the medium was removed and replaced with medium containing either dopa (2.5 x 10^{-4}M), dopachrome (8 x 10^{-5}M), phosphate buffer, or buffer previously mixed with Ag_2O. After 2 hr of incubation, the medium was removed and cells were washed with fresh medium and reincubated for 24 hr. Cells were harvested as above and counted.

RESULTS

Thymidine incorporation studies

Effect of dopa and dopachrome - Wild type cells exposed to dopa or dopachrome simultaneously with ^{3}H-thymidine exhibited a reduction in the rate of incorporation of ^{3}H-thymidine into acid-precipitable material (Fig. 2). Dopachrome was clearly more effective in reducing this incorporation (c.p.m. / 10^5 cells) than dopa. After 4 hr of incubation with dopachrome, ^{3}H-thymidine incorporation was approximately 15% of the control value. By this time, many of the cells exposed to dopachrome had either lysed or were rounded and floating in the medium. On the other hand, cells exposed to dopa had the same morphology as the controls.

A dose-response curve was constructed by comparing the effects of various concentrations of dopa and dopachrome on ^{3}H-thymidine incorporation (Fig. 3). To achieve a 50% reduction in the incorporation of thymidine, about twenty times more dopa than dopachrome was required.

Effect of pretreatment with MSH - Following 48 hr of incubation with MSH and MIX, the wild type melanoma cells had a noticeable increase in melanin formation and their shape was more flattened with increased dendritic processes (Fig. 4 a,b). Similar observations have been made previously

(4). Treatment with MSH and MIX for 48 hr markedly decreased the incorporation of ^{3}H-thymidine into acid-precipitable material and the addition of dopachrome did result in a further decrease (Fig. 5). However, incubation with MSH/MIX resulted in such a degree of inhibition that the value could not be used as a sensitive control. Decreasing the time of exposure to MSH/MIX to 24 hr resulted in similar responses. Fortunately, a second assay, based upon cell counts taken 24 hr after exposure to melanin precursors, proved to be a more sensitive measure of cytotoxicity.

Cell viability studies

Effect of pretreatment with MSH - After 2 hr of incubation of wild type melanoma cells with dopachrome, the culture medium had turned dark. If cells had been previously treated with MSH and MIX, dopachrome caused them to round up and many were floating in the medium or were attached only by fine dendritic processes (Fig. 4 c,d). In contrast, cells without any pretreatment had the same morphology as control cells when exposed to dopachrome. The toxic changes which resulted from dopachrome and MSH/MIX treatment first began to appear at an incubation time of 60 min. On the other hand, the cells exposed to dopa had no change in morphology, regardless of pretreatment.

Figure 6 shows the effect of pretreatment with MSH/MIX on cellular growth inhibition 24 hr after exposure to dopa or dopachrome. Results are expressed as a percentage of the control cell count according to the formula (number of treated cells/number of control cells) x 100. On the average, there were 15% less cells as the control figure for the MSH-treated cells as compared to the non-MSH-treated cells.

Dopachrome alone was able to inhibit cellular growth to a greater extent than dopa, even though lower concentrations of dopachrome were employed (Fig. 6). Although no inhibition of growth was seen in cells exposed to dopa (1.2×10^{-4}M) for 2 hr, dopachrome (8×10^{-5}M) resulted in a cell count of 54% as compared to the control. In addition, exposure to dopachrome after treatment with MSH/MIX resulted in a marked decrease in cell number. A cell count of 54% was observed with dopachrome (8×10^{-5}M) alone, whereas a count of 21% was seen with the combination of MSH, MIX, and dopachrome. As previously described (44), a greater degree of growth inhibition was seen with dopa plus MSH/MIX than with dopa alone.

Two dose response curves were constructed by comparing the effects of various concentrations of dopa and dopachrome on the growth of either <u>mel-1</u> or <u>amel-1</u> cells (Fig. 7). Similar results were seen with the <u>amel-1</u> cells when they were counted 48 hr following exposure to dopa or dopachrome.

Dopachrome by itself was observed to be more toxic than dopa in both mel-1 and amel-1 cells (Fig. 7). Even with MSH/MIX pretreatment, dopa had no effect on the amel-1 cells. In both mel-1 and wild type cell cultures, a greater degree of growth inhibition was seen with dopa plus MSH/MIX than with dopa alone. Pretreatment with MSH/MIX also enhanced the growth inhibition of dopachrome in both mel-1 and amel-1 cells. In mel-1 cells, dopachrome resulted in a 50% reduction in cell number at a concentration of 3.5×10^{-4}M; if the cells were first treated with MSH/MIX, a concentration of 7×10^{-5}M had the same effect (Table 1). In mel-1 cells, the enhancement of dopachrome's cytotoxicity by MSH/MIX was more marked than in amel-1 cells and similar to that seen in wild type cells (Fig. 8). Following MSH/MIX treatment and exposure to dopachrome for 2 hr, a 50% reduction in cell count was observed at a concentration of 2.5×10^{-4}M in amel-1 cells and a concentration of 7×10^{-5}M in mel-1 cells (Table 1).

In one study, cell counts were done immediately following the 2 hr exposure to either dopa or dopachrome rather than 24 hr later. At this earlier time point, the number of cells attached in the dopa- or dopachrome-treated cultures was the same as in untreated control cultures.

Cell counts of the freely floating cells that were removed with the medium did not account for the differences in

the values for attached cells. This indicated that dopachrome caused cellular lysis rather than simply a detachment of the cells from the flask. The cells that did detach because of dopachrome treatment were not viable in that 90% of these cells were unable to exclude the dye trypan blue and could not reattach in fresh culture medium.

Individual effects of MSH, MIX, and dibutyryl cyclic AMP - Exposure to MIX (5×10^{-5}M) for 48 hr had no effect on dopachrome toxicity (Fig. 9). With MIX pretreatment, the cell count was 76% of the control figure and with dopachrome alone, it was 71%. Incubation with MSH (1×10^{-7}M) led to a growth figure of 61% 24 hr after dopachrome exposure, while the combination of MSH/MIX resulted in a cell count of 48%. Although MIX by itself had no effect on growth inhibition, it did potentiate the effects of MSH.

When the cells were exposed to dibutyryl cyclic AMP (1 mM) for 48 hr, then dopachrome (8×10^{-5}M) for 2 hr, they exhibited growth inhibition comparable to that seen with MSH/MIX pretreatment (Fig. 10). Cell counts were 39% with dibutyryl cyclic AMP pretreatment and 30% with MSH/MIX pretreatment. In the case of treatment with dopa, dibutyryl cyclic AMP resulted in more marked cellular inhibition than MSH/MIX.

DISCUSSION

In the present studies, dopachrome was shown to be a melanin precursor with potent toxic effects on S-91 melanoma cells. Dopachrome's cytotoxic properties were reflected in both decreased thymidine incorporation (Fig. 2,3) and increased growth inhibition (Fig. 7). In both MSH- and non-MSH-treated cells, dopachrome was more effective than either dopa or Ag_2O-treated buffer. For example, about twenty times more dopa than dopachrome was required to achieve a 50% reduction in the incorporation of thymidine (Fig. 3).

Further investigations supported the hypothesis that pretreatment of melanoma cells with MSH would increase the cytotoxicity of dopachrome. When the exposure of wild type cells to dopachrome was preceded by incubation with MSH/MIX, a reduction in cell count from 54% (dopachrome alone) to 21% was seen (Fig. 6). A similar degree of growth inhibition was seen in <u>mel-1</u> cells and to a lesser extent in <u>amel-1</u> cells (Fig. 8). Following incubation with MSH/MIX for 48 hr and exposure to dopachrome for 2 hr, a 50% reduction in cell count was observed at a concentration of $2.5 \times 10^{-4}M$ in <u>amel-1</u> cells and a concentration of $7 \times 10^{-5}M$ in <u>mel-1</u> cells (Table 1).

The above results provide evidence for a more distal site of regulation in the pathway of melanin synthesis than

that of tyrosinase. First of all, dopachrome's further conversion was independent of tyrosinase and yet its cytotoxicity was increased by pretreatment with MSH. Secondly, this increased toxicity was seen in the amel-1 cells, which have negligible tyrosinase activity (50). Parallel studies in this laboratory showed that extracts of non-MSH treated cells contain an inhibitory factor which blocks the spontaneous conversion of dopachrome to melanin, and pretreatment of cells with MSH removed this factor (28). The results presented here suggest that this inhibitory factor which blocks melanogenesis also protects the cells from the toxicity of melanin precursors.

Based upon the studies described here, cyclic AMP is a likely agent in the removal of the inhibitory factor by MSH. Dibutyryl cyclic AMP, an analogue of cyclic AMP, was able to mimic the effects of MSH/MIX on growth inhibition of melanoma cells. After a 2 hr exposure to dopachrome, a cell count of 30% was seen 24 hr later if cells were pretreated with MSH/MIX and a count of 39% if dibutyryl cyclic AMP (1 mM) was the pretreatment (Fig. 10). This particular concentration of dibutyryl cyclic AMP was chosen because it results in the same cellular changes as MSH - an increase in pigmentation and dendritic processes and a marked slowing of cellular proliferation (56).

Once evidence for an inhibitory factor was provided, the

next step was to localize the point at which the inhibition was exerted. One clue was the observation that if extracts of non-MSH treated cells (presumably containing the factor) were added to dopachrome, the solution turned from orange to colorless and remained colorless for 24 hr (Fig. 1). Therefore, the point of regulation was beyond the conversion of dopachrome to 5,6-dihydroxyindole, the step catalyzed by the factor "DCF" (27). As a further step, the reaction products resulting from the mixture of dopachrome and extract from non-MSH treated cells were examined by high pressure liquid chromatography. The major component was 5,6-dihydroxyindole (28), making the conversion of 5,6-dihydroxyindole to indole-5,6-quinone the most likely point of inhibition.

As a result of the MSH-controlled inhibitory factor, 5,6-dihydroxyindole would accumulate in non-MSH treated cells, while indole-5,6-quinone would be formed in MSH treated cells. Therefore, the increased cell killing seen in MSH treated cells is most likely secondary to the increased intracellular concentration of indole-5,6-quinone. Quinones are known sulfhydryl group scavengers, thus inhibiting key enzymes in DNA synthesis (38) and cellular respiration (37). It seems likely therefore that the quinone intermediates are the toxic precursors in the melanin pathway.

Beyond the interest generated by the possibility of a

second MSH-controlled regulation point, the implications for chemotherapy of human melanoma must be considered. In the studies described here, incubation of melanoma cells with MSH/MIX for 48 hr and subsequent exposure to dopachrome (8×10^{-5}M) for only 2 hr resulted in a cell count which was 20% of the control value, i.e. a growth inhibition of 80%. In a similar *in vitro* investigation by Wick *et*. *al*., dopa (6 mM) resulted in a growth inhibition of 60% in melanotic S-91 cells (45) and 6-hydroxydopa (10 ug/ml) an inhibition of 75% (46). In the present studies, however, dopa alone had little effect on cellular growth (Fig. 6). This discrepancy can be explained by the fact that Wick *et*. *al*. used about 20-40 times more dopa in their experiments. As compared to dopa (6 mM), dopachrome produced more inhibition at 1/100th the concentration.

There has been, however, an obvious lack of correlation between *in vitro* toxicity and *in vivo* toxicity. For instance, when 6-hydroxydopa was tested against B-16 and S-91 melanoma *in vivo*, no significant prolongation in the survival of treated animals was seen (46). In studies with dopa, this melanin precursor did result in a slight (20%) prolongation of the survival of mice bearing B-16 melanoma (57) and enhancement of L-dopa incorporation into melanoma *in vivo* has been accomplished by inhibiting dopa decarboxylase (58). However, a temporal relationship has been reported between

the initiation of L-dopa therapy for Parkinsonism and growth of melanoma (59,60,61). These observations make the testing *in vivo* of the combination of MSH and dopachrome imperative prior to making further claims regarding its application to chemotherapy of melanoma.

In conclusion, a second MSH-controlled point of regulation in the melanin pathway has been suggested by cytotoxicity studies. Further investigations are being conducted to isolate and identify this particular inhibitory factor. In addition, the *in vivo* cytotoxicity of dopachrome when coupled with MSH pretreatment is being tested. Perhaps these findings will bear favorably upon the future chemotherapy of human melanoma. The toxicity of melanin precursors would be utilized in this form of chemotherapy "in which the color of melanin indeed becomes a blush of metabolic embarrassment" (29).

<u>Figure 1</u>: The classical pathway of melanin biosynthesis, modified slightly from the scheme of Mason and Raper (62).

Figure 1

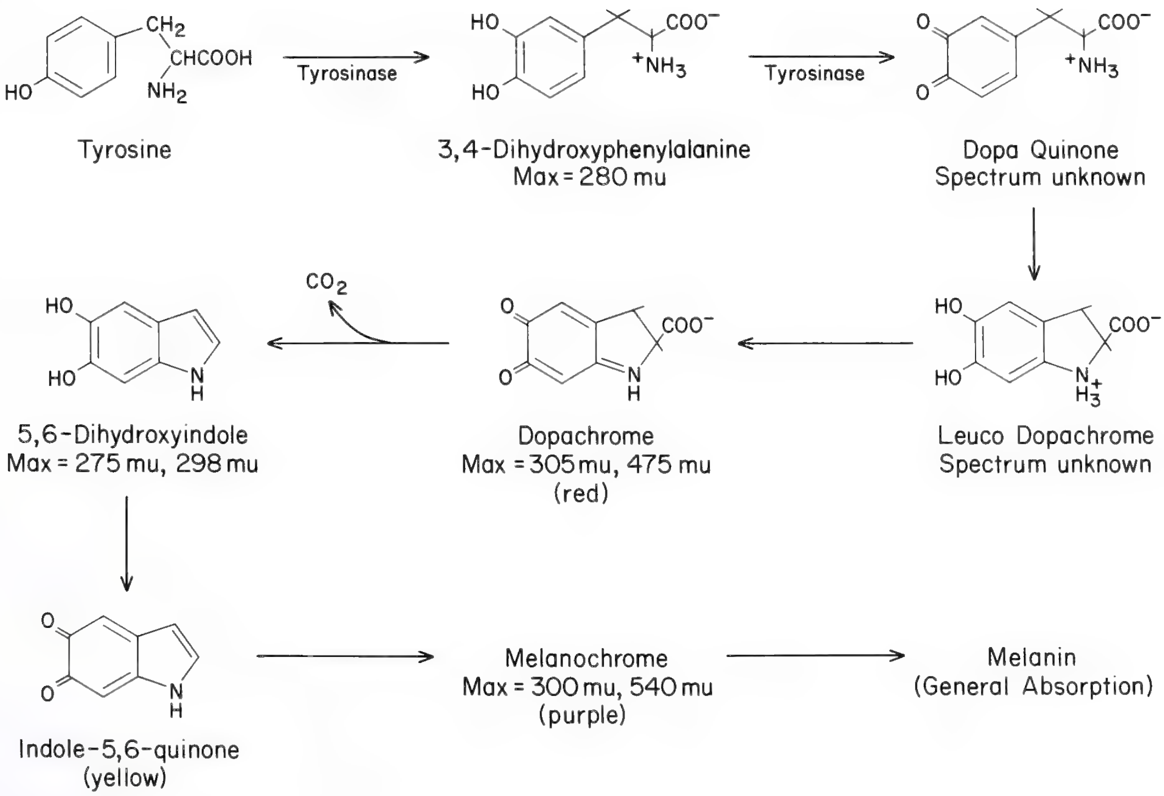
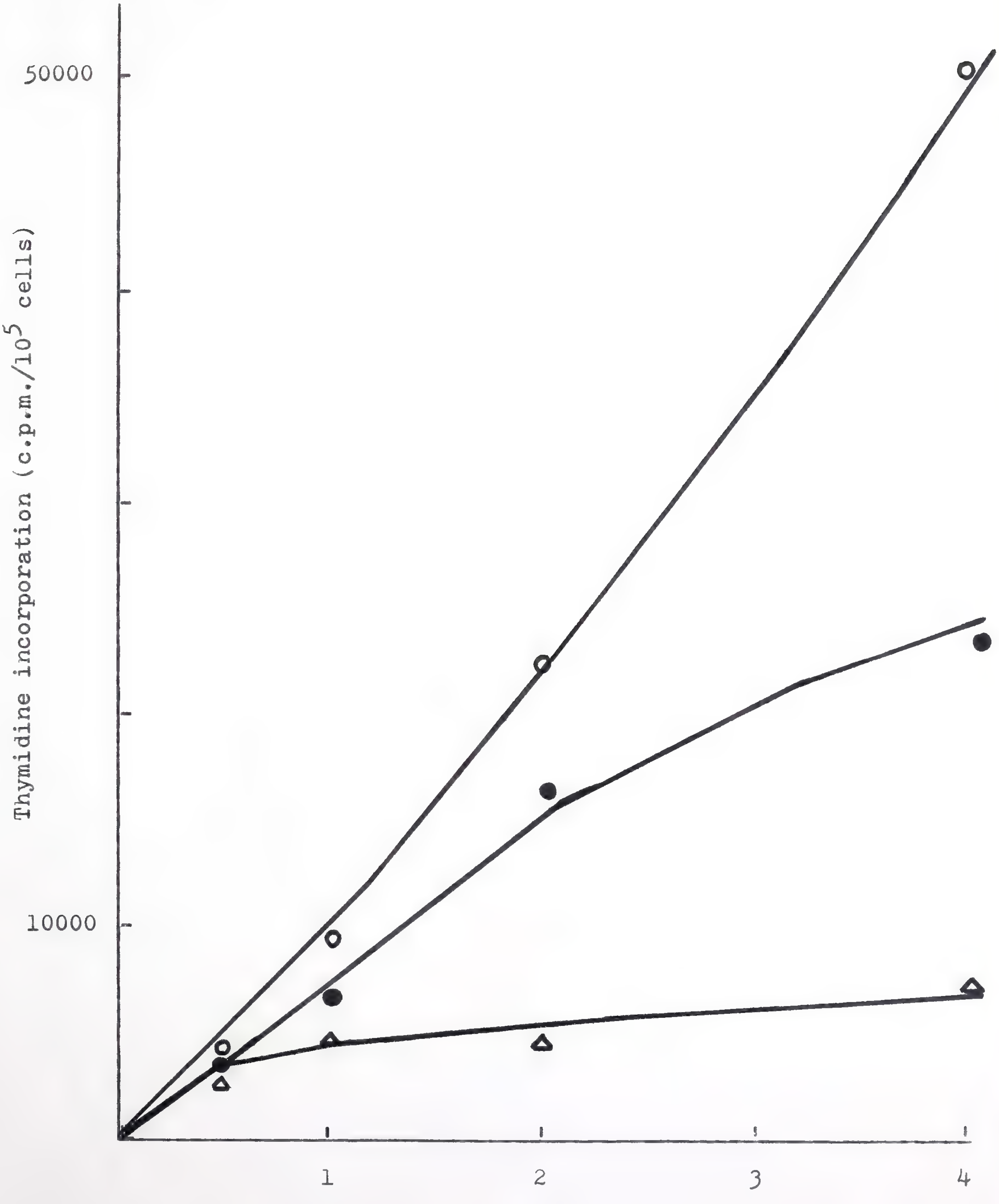

Tyrosinase
Tyrosinase
Tyrosine
3,4-Dihydroxyphenylalanine
Max = 280 mu
Dopa Quinone
Spectrum unknown
CO_2
5,6-Dihydroxyindole
Max = 275 mu, 298 mu
Dopachrome
Max = 305 mu, 475 mu
(red)
Leuco Dopachrome
Spectrum unknown
Indole-5,6-quinone
(yellow)
Melanochrome
Max = 300 mu, 540 mu
(purple)
Melanin
(General Absorption)

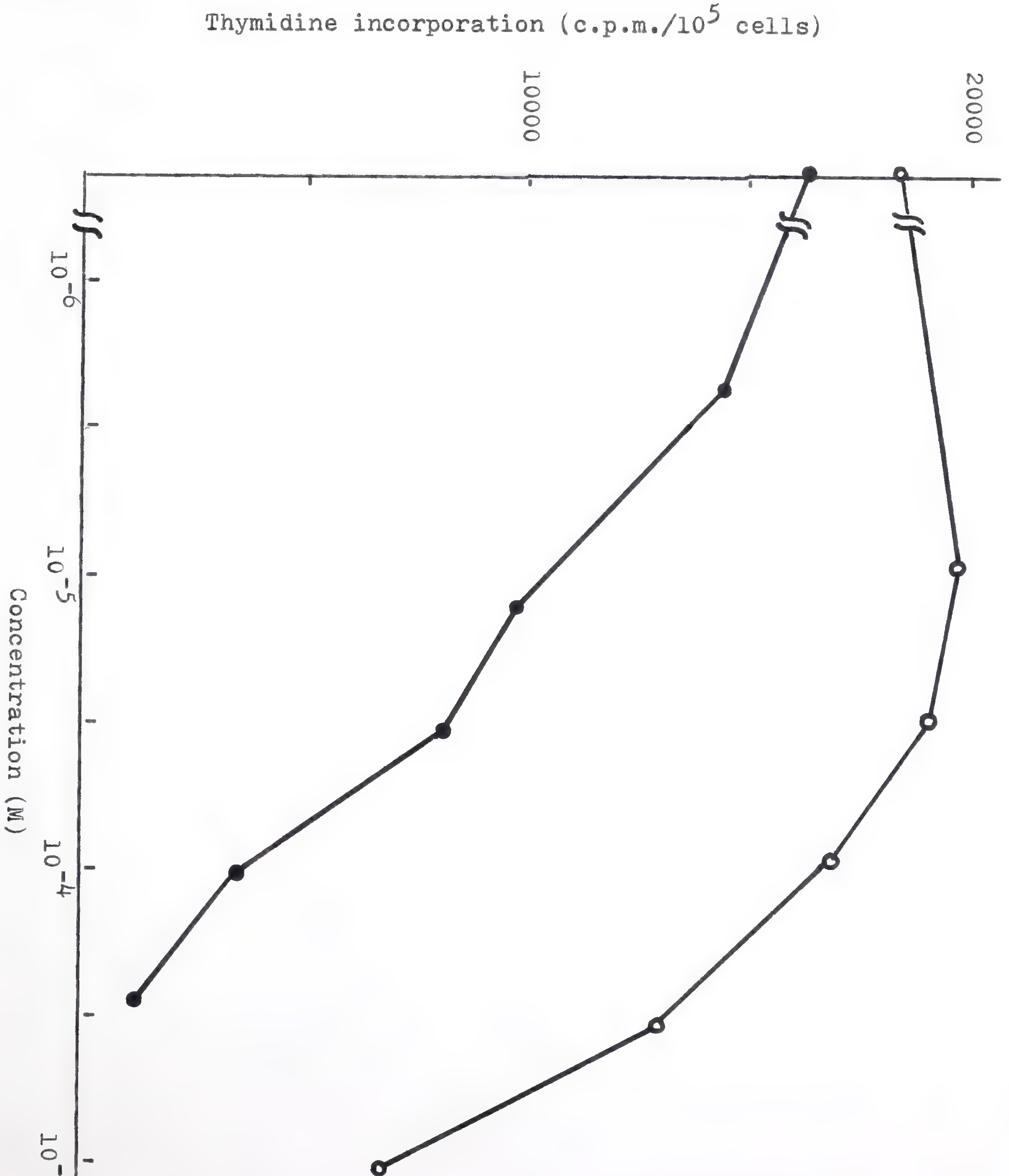
Figure 2: Effects of dopa and dopachrome on the rate of thymidine incorporation into acid-precipitable material in wild type melanoma cells. At the times indicated the amount of ^{3}H-thymidine incorporation into acid-precipitable material was determined in triplicate culture flasks. Variation was less than ± 15%. The experiment was repeated once, with similar results. ○, phosphate buffer; ●, dopa (2.5 x 10^{-4}M); △, dopachrome (7 x 10^{-5}M).

Figure 2


50000
10000
Thymidine incorporation (c.p.m./10^5 cells)
1
2
3
4
Time (hr)

<u>Figure 3</u>: Effects of various concentrations of dopa and dopachrome on thymidine incorporation into acid-precipitable material in wild type melanoma cells. Dopa (○) and dopachrome (●) were added at the concentrations indicated. After 4 hr the amount of ^{3}H-thymidine incorporation into acid-precipitable material was determined in triplicate flasks. Variation was less than ± 15%. The experiment was repeated once, with similar results.

Figure 3


Thymidine incorporation (c.p.m./10^5 cells)
10000
20000
10^{-6}
10^{-5}
10^{-4}
10^{-3}
Concentration (M)

<u>Figure 4</u>: Phase contrast photographs of wild type melanoma cells grown for 48 hr in normal medium (left) or medium containing MSH/MIX (right). Upper: cells were exposed to phosphate buffer for 2 hr; lower: cells were exposed to dopachrome (8×10^{-5}M) for 2 hr.

Figure 4

Figure 5: Effect of pretreatment with MSH/MIX on thymidine incorporation into acid-precipitable material in wild type melanoma cells. Following 48 hr of growth in medium containing either no additions (□) or MSH/MIX (▨), cells were exposed to phosphate buffer or dopachrome (4 or 8 x 10^{-5}M) simultaneously with ^{3}H-thymidine. After 2 hr the amount of ^{3}H-thymidine incorporation into acid-precipitable material was determined in triplicate flasks. Variation was less than ± 15%. The experiment was repeated three times, with similar results.

Figure 5

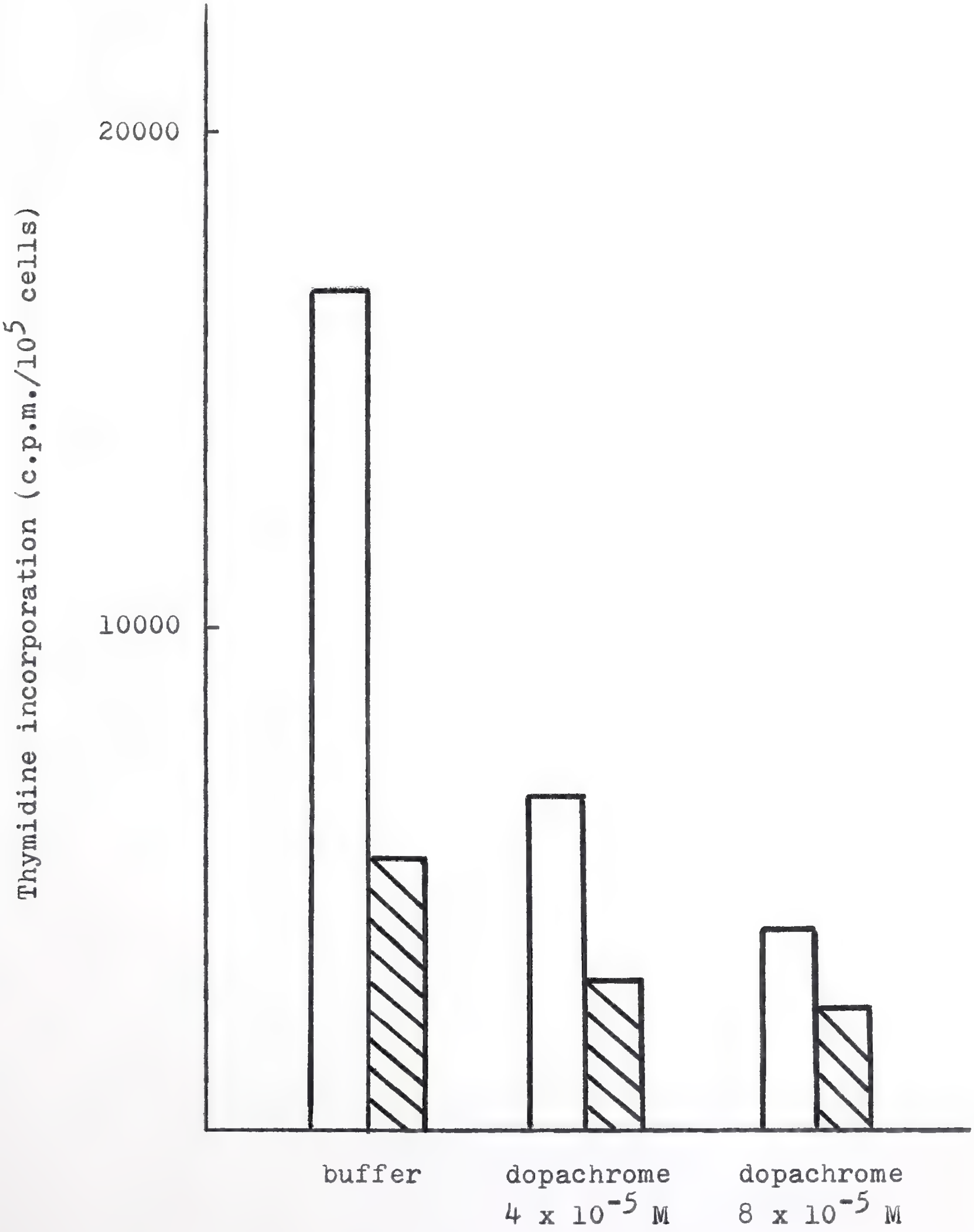

Thymidine incorporation (c.p.m./10^5 cells)
20000
10000
buffer
dopachrome
4 x 10^{-5} M
dopachrome
8 x 10^{-5} M

Figure 6: Effect of pretreatment with MSH/MIX on the viability of wild type melanoma cells following exposure to dopa or dopachrome. Following 48 hr of growth in medium containing either no additions (▭) or MSH/MIX (▨), cells were exposed to phosphate buffer, dopa (1.2 or 2.5 x 10^{-4}M), or dopachrome (4 or 8 x 10^{-5}M) for 2 hr. After 24 hr cell counts of triplicate flasks were made. Variation was less than ± 15%. The experiment was repeated once, with similar results.

Figure 6

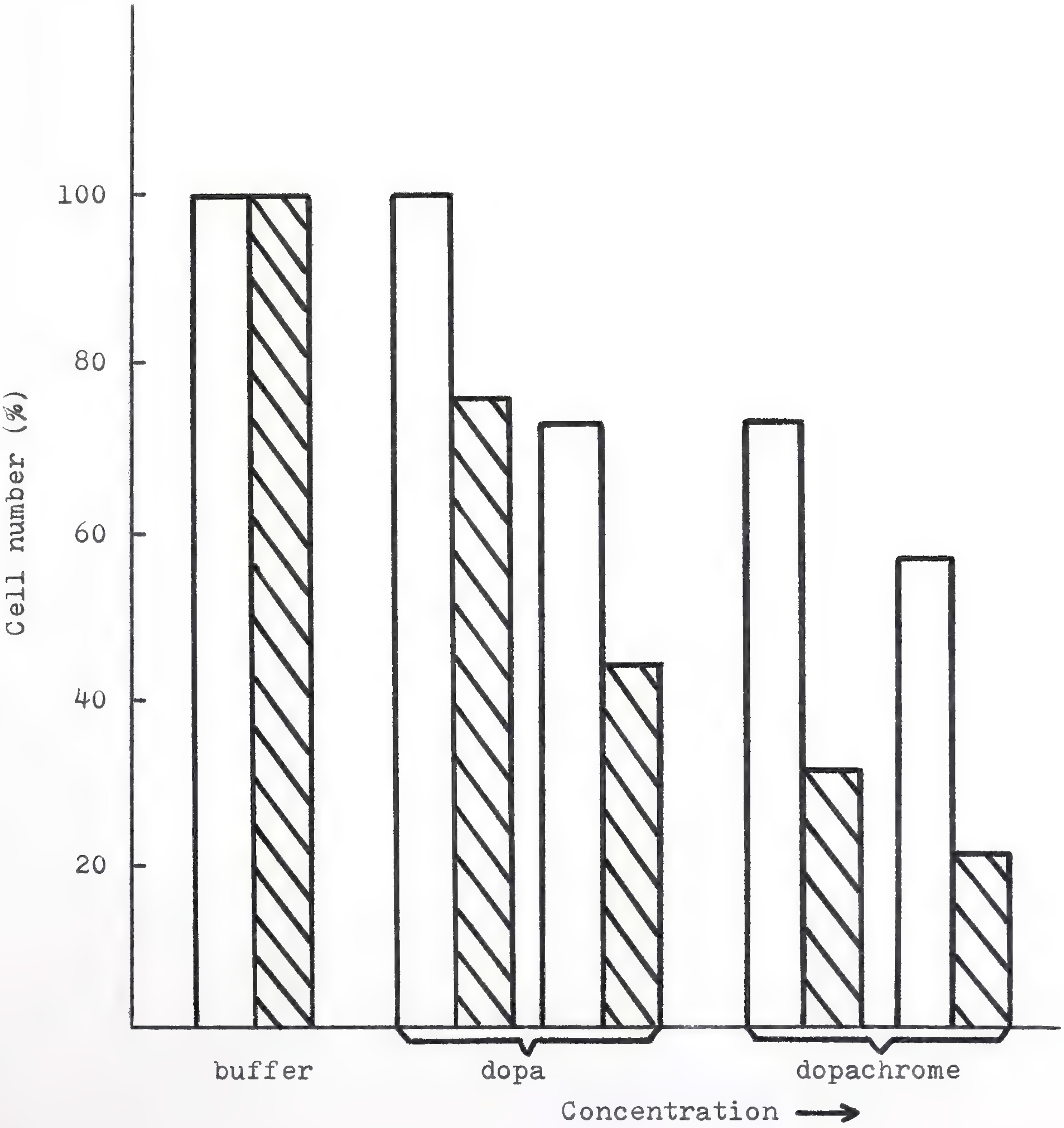

Cell number (%)
100
80
60
40
20
buffer
dopa
dopachrome
Concentration →

Figure 7: Effect of pretreatment with MSH/MIX on the toxicity of dopa and dopachrome against mel-1 and amel-1 melanoma cells. Following 48 hr of growth in medium containing either no additions or MSH/MIX, cells were exposed to dopa or dopachrome at the concentrations indicated for 2 hr. Control cultures received either buffer that had been mixed with Ag_2O and filtered or buffer alone. After 24 hr cell counts of quadruplicate flasks were made. Variation was less than ± 10%. Limited versions of these experiments were repeated twice, with similar results.

Figure 7

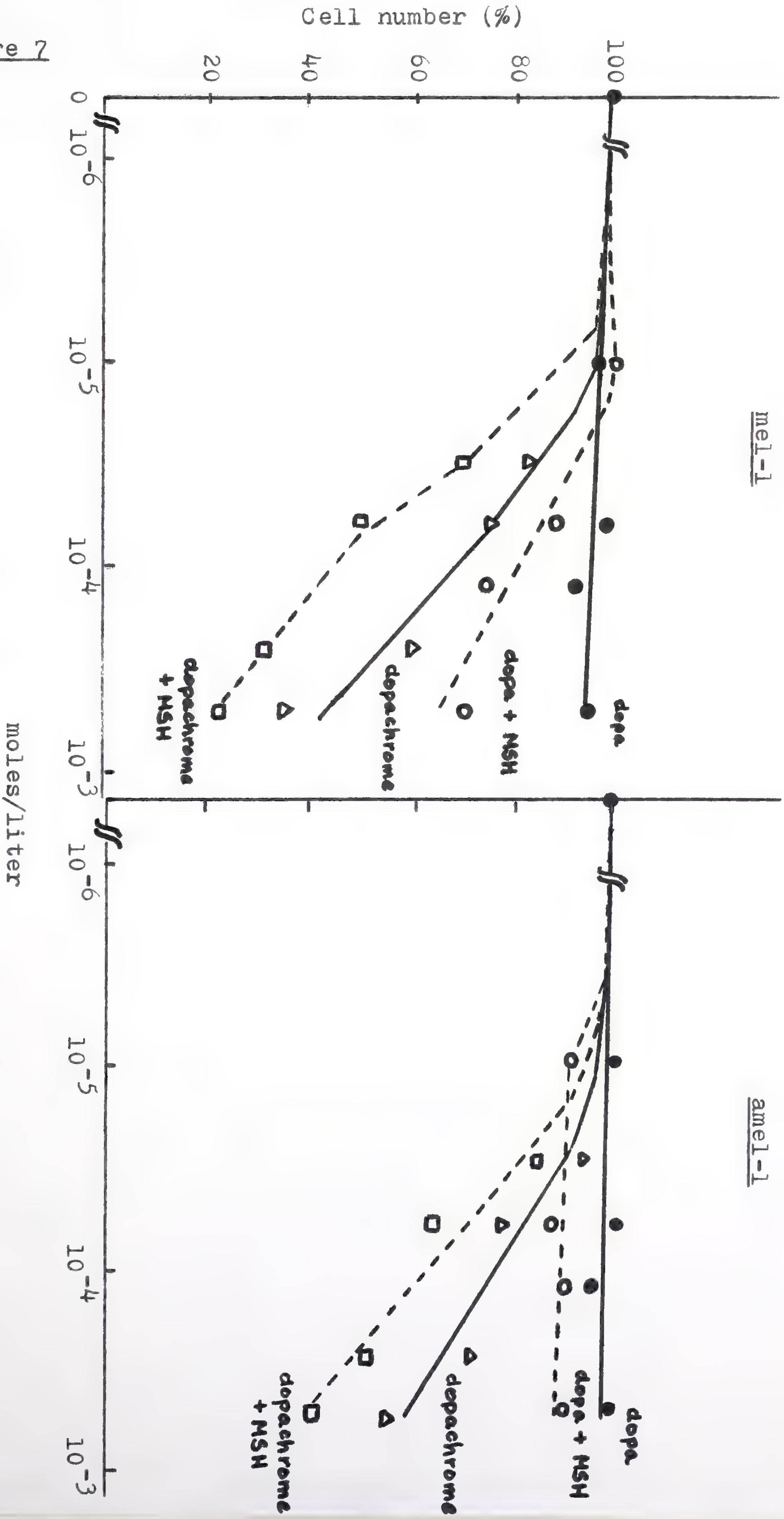

Cell number (%)
0
20
40
60
80
100
moles/liter
10^{-6}
10^{-5}
10^{-4}
10^{-3}
mel-1
amel-1
dopa
dopa + MSH
dopachrome
dopachrome + MSH

Figure 8: Effect of preincubation with MSH/MIX on the toxicity of dopachrome in three melanoma cell lines. Following 48 hr of growth in medium containing either no additions (▭) or MSH/MIX (▨), wild type, *mel-1* and *amel-1* melanoma cells were exposed to dopachrome (4 or 8 x 10^{-5}M) for 2 hr. After 24 hr cell counts of triplicate flasks were made. Variation was less than ± 10%. The experiment was repeated once, with similar results.

Figure 8

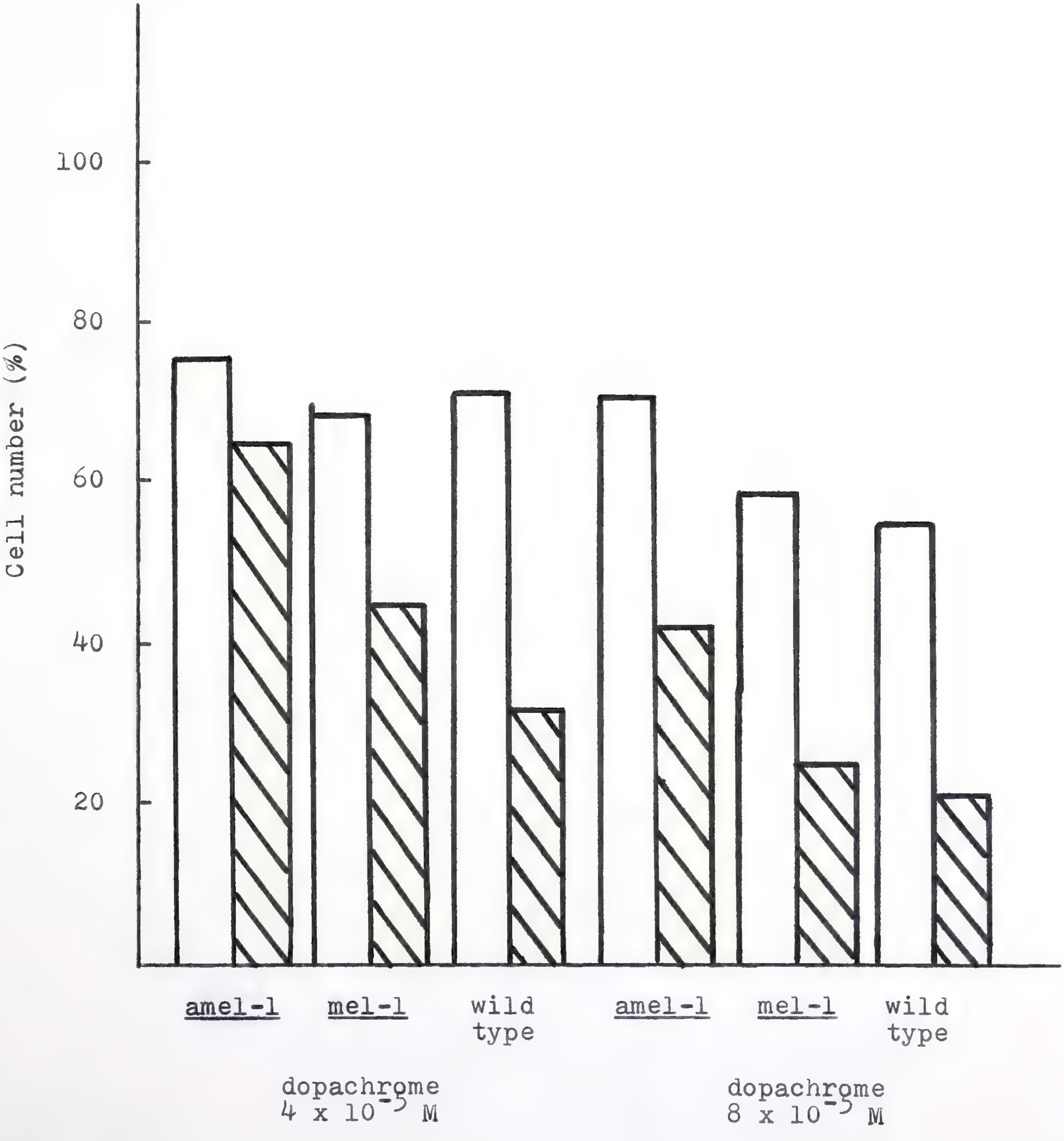
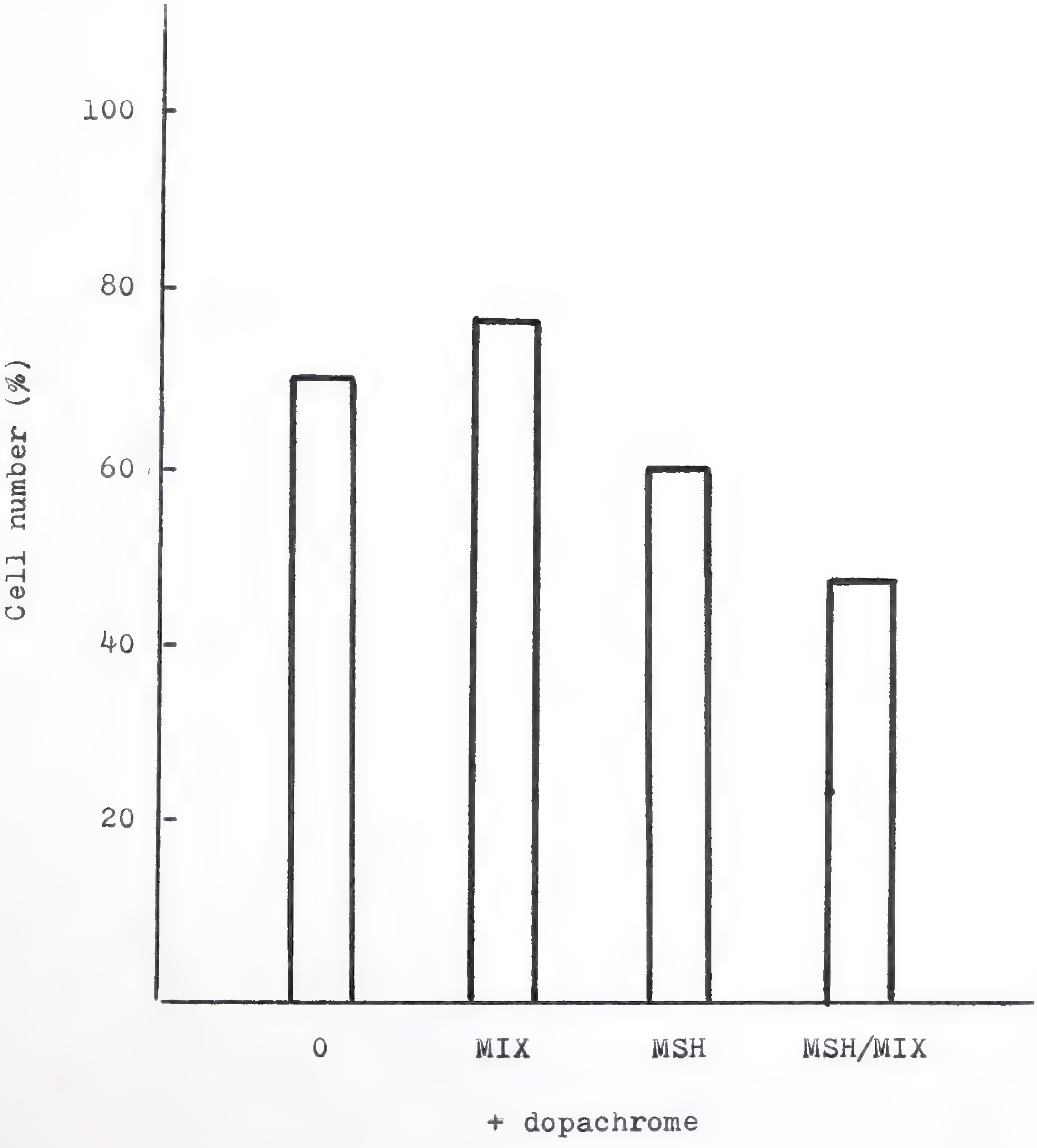

100
80
60
40
20
Cell number (%)
amel-1
mel-1
wild type
amel-1
mel-1
wild type
dopachrome 4 x 10^{-5} M
dopachrome 8 x 10^{-5} M

Figure 9: Effect of preincubation with MSH versus MIX on the toxicity of dopachrome. Following 48 hr of growth in medium containing MSH, MIX, MSH/MIX, or no additions, cells were exposed to dopachrome (8×10^{-5}M) for 2 hr. After 24 hr cell counts of triplicate flasks were made. Variation was less than ± 10%. The experiment was repeated once, with similar results.

Figure 9



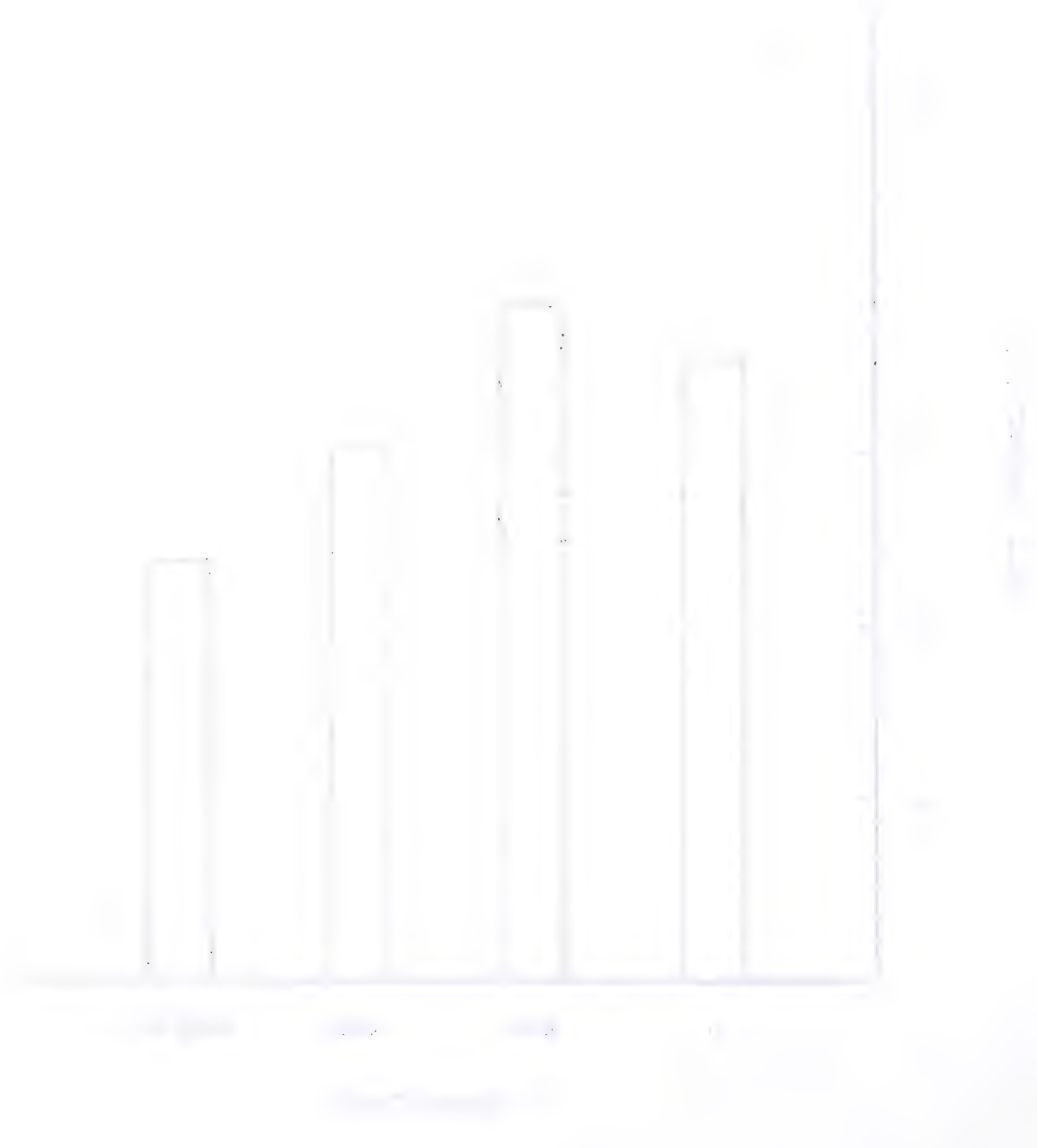


Figure 10: Effect of preincubation with MSH/MIX versus dibutyryl cyclic AMP on the cytotoxicity of dopa and dopachrome in melanoma cells. Following 48 hr of growth in medium containing MSH/MIX (▧), dibutyryl cyclic AMP (▨), or no additions (▤), cells were exposed to dopa (2.5 x 10^{-4}M) or dopachrome (8 x 10^{-5}M) for 2 hr. Control cultures received either buffer that had been mixed with Ag_2O and filtered or buffer alone. After 24 hr cell counts of triplicate flasks were made. Variation was less than ± 10%.

Figure 10

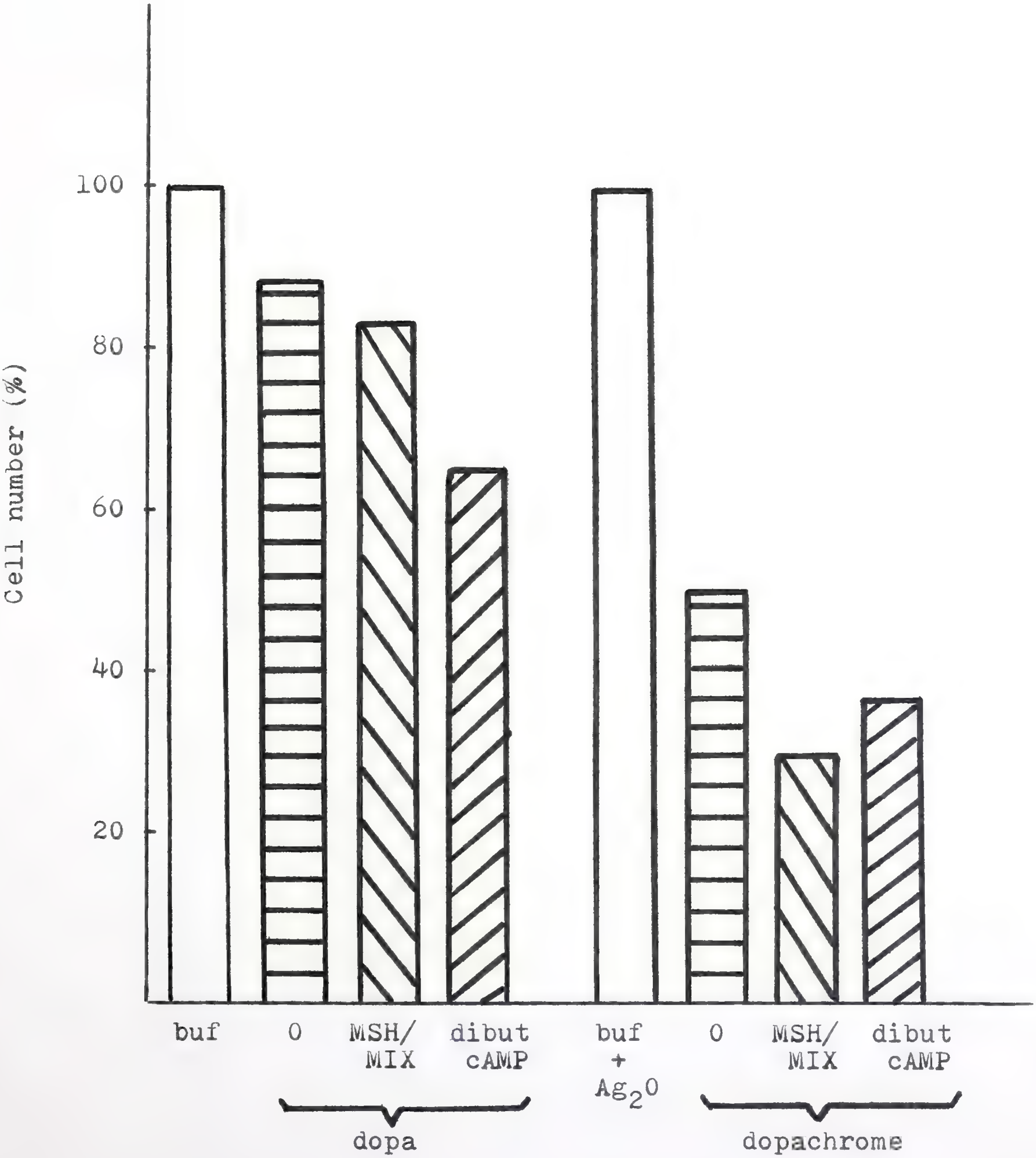

Cell number (%)
100
80
60
40
20
buf
0
MSH/ MIX
dibut cAMP
dopa
buf + Ag_2O
0
MSH/ MIX
dibut cAMP
dopachrome

Table 1: Concentration of dopa and dopachrome required for 50% reduction in cell number

Treatment	Concentration for 50% Reduction	
	MSH-responsive Cells	Non-responsive Amelanotic Variants
None	-----	-----
Dopa	-----	-----
Dopa + MSH	1.5×10^{-3}M	-----
Dopachrome	3.5×10^{-4}M	4.5×10^{-4}M
Dopachrome + MSH	7×10^{-5}M	2.5×10^{-4}M

*The experimental design is described in the legend to Fig. 7.

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